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Original Research Article

THE EFFECT OF ANTIBIOTIC /ANTISEPTIC COMBINATION ON *PROPIONIBACTERIUM ACNES* TO OVERCOME ANTIBIOTIC RESISTANCE.

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Abstract:

Background: Acne vulgaris has been treated usually with antibiotics. *Propionibacterium acnes* (*P. acnes*) play a crucial role in its pathogenesis, however, *P. acnes* has been described to develop antibiotic resistance.

Objective: To assess the antibacterial effect of certain antiseptics such as hydrogen peroxide, apple cider vinegar and honey in combination of specific antibiotic on *P. acnes*, to decrease its resistance.

Method: Acne samples were cultured under anaerobic condition to isolate and identify *P. acnes*, using microscopic investigations, biochemical tests and molecular identification. Susceptibility tests of the isolated *P. acnes* against various antibiotics were performed using disc diffusion and Minimum inhibitory concentrations (MICs) assays. Antibiotic/antiseptic combination effect was determined using checkerboards analysis.

Results: *P. acnes* were isolated from 68 out of 186 specimens (36.5%). Resistance of *P. acnes* isolates to Clindamycin is increasing (72.25%), although *P. acnes* showed susceptibility to other antibiotics. Hydrogen peroxide/Clindamycin Combination showed synergism in 83.3% of the isolates, with a fractional inhibitory concentration index (FICI) of 0.348. While, combination of Clindamycin with honey or apple cider vinegar give synergism in 25% and 2.9% of the isolates respectively .

Conclusion: The combination of hydrogen peroxide with Clindamycin has potential *in vitro* activity to overcome Clindamycin resistance in *P. acnes*.

Keywords: Acne, *P. acnes*, antibacterial resistance, antibiotic/antiseptic combination effect

Introduction:

Acne vulgaris is a common inflammatory disease of pilosebaceous follicles that affects nearly 85% of young adults and adolescents (1). *Propionibacterium acnes* plays a crucial role in the pathogenesis of acne vulgaris (2). Pathogenesis of acne including a complex interplay of bacterial colonization, inflammation and increased sensitivity to androgens circulating in blood (3). *P. acnes* is a gram-positive polymorphic, facultative anaerobic, slow-growing and nonmotile bacillus (4). The current study was conducted using both conventional and molecular identification of *P. acnes*, as molecular techniques provide quick, powerful, reliable, and sensitive tool in identification of such fastidious organisms (5).

P. acnes can cause opportunistic infections that harm the host through secretion of certain extracellular enzymes including hyaluronidase, lipase and protease (6). The ability of *P. acnes*

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to secrete hyaluronidase enzyme and degrade hyaluronic acid to penetrate tissues rich in hyaluronic acid, is one of its major virulence factors (7).

Specific antibiotics such as Clindamycin, erythromycin and tetracycline are used extensively for treatment of acne vulgaris, which lead to the emergence of drug resistance of *P. acnes* strains against these antibiotics, over the past 30 years (8,9).

This study was undertaken to examine the bacteriological profile of *P. acnes* and to ascertain its antimicrobial resistance patterns. Additionally, We assessed the effect of specific compounds of known antimicrobial activity such as honey, vinegar and hydrogen peroxide (10–12), alone and in combination with Clindamycin, as one of the highest used antimicrobial compound, against which *P. acnes* developed resistance (9).

2- Materials and Methods:

2.1- Patients:

The present study involved one hundred fifty patients with acne vulgaris attending the dermatology outpatient clinic of El-Hussein Hospital, Cairo-Egypt; Over a period of five years between November 2014 to July 2017. All patients were included after getting the informed consent. The study protocol was reviewed and approved by Ethical Committee of El-Hussein Hospital, Al-Azhar University, Cairo-Egypt. All demographic data (including sex, age, history of antibiotic treatment, time of disease's onset) were collected at the time of patient enrolment.

Samples were collected by dermatologist from closed comedones (whiteheads) and open comedones (blackheads) using a sterile (25 × 35 mm) hypodermic needle and comedone extractor under aseptic condition.

2.2- Bacteriological study

Samples were transported using cooked meat broth (Hi media, Mumbai, India). *Samples* were inoculated on reinforced clostridium agar (Oxoid, UK)(13), and incubated under anaerobic conditions using the Gaspak system (HiMedia Labs., Mumbai, India), at 37°C for 3 day. The samples were also inoculated on manitol salt agar (Oxoid, UK) and incubated under aerobic conditions at 37°C for 24hr.

2.2.1 Phenotypic identification

The growing cultures were identified using colony morphology, Gram stain, and certain biochemical tests. The presumptive identification of *P. acnes* was done as anaerobic Gram-positive rods with positive oxidase, indole, catalase, glucose and maltose fermentation and motility test (4,14). To confirm the conventional identification of *P. acnes*, molecular identification of 16s-rRNA of *P. acnes* and Hyaluronidase enzyme gene using PCR were carried out.

2.2.2 Molecular identification of *Propionibacterium acnes*

A total volume of 300 µL of the isolated bacteria were placed in 200 µL of Tris-EDTA buffer (TE buffer); then 2 µL of lysozyme were added and kept for 30 min. at room temperature. Extraction of DNA was then performed following the extraction kit's instructions (QIAGEN, Germany).

Following the instruction of QIAGEN kit (QIAGEN, Germany), 20µL PCR mix was prepared, containing: Master Mix (10µL), forward and reverse primers 0.5 µL of each (10 pmol), the

extracted DNA (5µL), and sterile distilled water (4µL). The used primers synthesized by Thermo Fisher Scientific (Invitrogen). The PCR reactions were carried out for detection of 16s-rRNA gene and Hyaluronidase enzyme gene of *P. acnes*, using a thermocycler (Boeco, Germany). The used primer sequences, the expected product size and references of the used condition of PCR were listed in table 1. *P. acnes* standard strain (ATCC 6919), was used as a positive control. The products were then electrophoresed using agarose gel (1.5%) and examined using UV light.

Table1: Target genes, primer sequences and expected product sizes

Gene	Primer	sequence	expected product size	Reference
16s-rRNA	Pf	5'-AGC TCG GTG GGG TTC TCT CAT C-3'	1202 bp	(Negin and Masood, 2017)
16s-rRNA	Pr	5'-GCT TCC TCA TAC CAC TGG TCA TC-3'	1202 bp	(Negin and Masood, 2017)
Hyaluronidase	Pf	5'-TTTCGGGATCCTTGTGTA- 3'	540 bp	(Harmony and Robin, 2015)
Hyaluronidase	Pr	5'-TCTGGACAACAAACCTGT-3'	540 bp	(Harmony and Robin, 2015)

2.3. Antibiotic susceptibility:

Antibiotic susceptibility of *P. acnes* was performed against Tetracycline (30µg), doxycycline (30µg), clindamycin (2µg), erythromycin (15µg) and clarithromycin (5µg); all purchased from Oxoid, UK; Clindamycin (Alexandria, Egypt), meropenem 10µg (Adwia, Egypt), Sulbactam plus ampicillin 20µg (Pfizer, Egypt), Benzyl-penicillin 10 unit (Misr, Egypt), Cefotaxim 30µg (Eipico, Egypt). The test was carried out using disc diffusion method on Mueller-Hinton agar (Oxoid Ltd., England). Additionally, broth microdilution assay, (MIC) was also performed. The data were interpreted using Clinical and Laboratory Standards Institute (CLSI, 2018) and European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2016). Inoculum suspension were prepared to match a 0.5 MacFarland standard. Antimicrobial agent stock solutions were prepared at concentrations of (2048 µg/mL) (15).

The MICs of honey stock conc. 100% v/v, hydrogen peroxide (10%v/v), and apple cider vinger 5% v/v against *P. acnes* was determined using the microdilution assay. 100 µL of Muller hinton broth (MHB) were added to all wells, except for Column number 12, in which 200 µL of the broth was included as a sterility control.

2.4. Determination of Fractional Inhibitory Concentration Index

The antibacterial potency of combination of Clindamycin with honey, vinger or hydrogen peroxide against *P. acnes* were evaluated using microdilution checkerboard assay to determine fractional inhibitory concentration index for each combination (16).

The combination was considered to have a synergistic effect when FICI value < 0.5, Additive when FICI value of 0.5 – 4 and Antagonism when FICI value of > 4.

2.5 statistical analyses

Statistical analyses were carried out using GraphPad Prism 6 software (GraphPad Software,

San Diego, CA, USA). Fisher's exact test was used to compare between categorical variables. Variables were described by number and percentage (N, %), and Continuous variables were compared using the Mann-Whitney U test.

3. Results:

The demographic data of acne patients showed that all of our acne patients aged from 11-35 years old, (mean 23.7). The majority (52.78%) of the patients were between 16-20 years old (Table2). *P. acnes* was isolated mostly (50%), from patients aged between 16-20 years.

Table 2: Age distribution of patients with acne vulgaris

	Age(years)	No. of cases	%
gp1	11-15	21	13.89
gp2	16-20	79	52.78
gp3	21-25	33	22
gp4	26-30	9	6
gp5	31-35	8	5.33

3.1. The percentage of the isolates identified using culture methods:

Of the total 186 specimens, 228 pathogenic organisms were isolated due to presence of mixed infection in some specimens. The isolated bacteriological identities are shown in Table 3. These pathogenic organisms include *P. acne*, *St. aureus*, *St. epidermidis*, *Peptostreptococcus*, *Peptococcus*, *Clostridium* and *Fucibacterium*. The microscopic appearance and the preceded biochemical tests results revealed that *P. acne* was one of the highest isolated pathogenic organism n=53/186 (28.49%) causing acne vulgaris.

Table 3. The isolated bacteriological profiles

Cocci	Rods
<i>St. aureus</i> 31 (13.59%)	<i>P. acne</i> 53 (28.49%)
<i>St. epidermidis</i> 34 (14.9%)	<i>Clostridium</i> 19 (8.3%)
<i>Peptostreptococcus</i> 72 (31.57%)	<i>Fucibacterium</i> 6 (2.6%)
<i>Peptococcus</i> 13 (5.7%)	

3.1.1. Phenotypic identification of *P. acnes*

The result of culture 186 samples of acne vulgaris using conventional anaerobic and aerobic culture technique and microscopic examination, showed that 79 samples out of 186 gives the characteristic appearance of *P. acnes*, which is pleomorphic rods, "Y" or "V" configurations, with a Chinese character" appearance arranged singly or in clumps. Afterward, for these 79 samples, certain biochemical tests were performed to confirm the identification of *P. acnes*, including the oxidase test, the catalase test, the indole, the motility test, and glucose and maltose fermentation test.

3.2. Molecular detection for identifying *P. acnes*:

In order to confirm the result of ordinary culture method for identification of *P. acne*, polymerase chain reaction (PCR) methods were carried out for identification of 16Sr-RNA gene. The results revealed that 68 isolates were positive for amplification of 16S- rRNA gene of *P. acnes*.

The present study used the culture technique and PCR to identify *P. acnes* in samples isolated from acne lesions. Of the total of 186 samples examined, 53 cases 28.5% were identified as *P. acnes*-positive through the culture method, while 68 cases 36.5% were identified as *P. acnes* using PCR. These results showing that PCR are much more accurate than conventional culture techniques.

3.2.1 PCR for identifying Hyaluronidase enzyme gene of *P. acnes*:

Polymerase chain reaction (PCR) methods for identifying *P. acnes* hyaluronidase enzyme gene revealed that 57 isolates were positive for amplification of hyaluronidase enzyme gene of *P. acnes*.

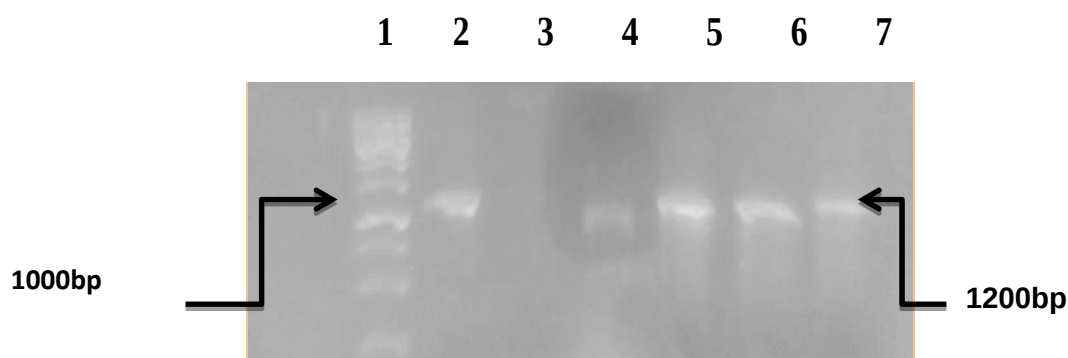


Fig 1: Amplification products electrophoresed on agarose gel (1.5%). 16 Sr-RNA. Well 1: 1-kb DNA ladder; Well

2: positive control *P. Acnes* standard strain (ATCC 6919); Well 3: negative control; Wells 4 to 7: *P. acnes*-positive samples

3.3. Antibiotic susceptibility pattern of *P. acnes* isolates:

The antibacterial screening was performed by disc diffusion to assess the sensitivity of *P. acnes* to tetracycline, erythromycin, doxycycline, Clindamycin, clarithromycin, sulbactam plus ampicillin, meropenem, benzyl-penicillin and cefotaxime against *P.acnes*.

Interpretation of the results was done according to European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2016) and Clinical and Laboratory Standards Institute (CLSI, 2018).

The antibacterial susceptibility was performed by disc diffusion and Broth microdilution assay, to assess the sensitivity of *P. acnes* isolates (n=68). The results showed high sensitivity to meropenem (78%), sulbactam plus ampicillin (75%), benzyl-penicillin (75%), cefotaxime (75%) On the other hand, *P.acnes* showed high resistance rates to Clindamycin (77.75%) and tetracycline (72.3%) Table 4 . All data were interpreted according to (EUCAST 2016) and (CLSI 2018) Table 4.

Table 4: MIC (µg/ml) results of *P.acnes* isolates (n=68) against specific antimicrobial agents

Antibiotic	MIC result/ml	Sensitive	
		no	%
Sulbactam plus ampicillin.	8-16	51	75
Cefotaxime.	2-32	51	75

Meropenem.	2-4	53	78
Benzyl-penicillin.	0.125-0.25	51	75
Clindamycin.	4-8	15	22.25
Doxycycline	4	36	52.7
Erythromycin	2-4	13	19.4
Tetracycline	16-32	19	27.7
Clarithromycin	0.25-0.5	30	44.5

3.4. Antiseptic susceptibility pattern of *P.acnes* isolates:

Antiseptic susceptibility pattern of *P. acnes* isolates against hydrogen peroxide, apple cider vinegar and honey was done using broth microdilution method. The data showed that around half of the candidate isolates were sensitive to 25% and 50% v/v of honey. Apple-cider-vinegar at Concentration 1.25% showed 62.5% sensitivity against *P. acnes*. Moreover, 0.15% hydrogen peroxide showed 70% sensitivity of *P. acnes* isolates.

Table 5: Susceptibility pattern of *P.acnes* isolates against honey, apple-cider-vinegar and hydrogen peroxide by broth microdilution method

Antiseptic	Sensitive							
	concentration	Sensitivity %	concentration	Sensitivity %	concentration	Sensitivity %	concentration	Sensitivity %
Honey	25% v/v	50	50% v/v	45	12.5%v/v	5	----	--
apple-cider-vinegar	1.25% v/v	62.5	0.0097% v/v	13	0.625% v/v	12.5	0.156% v/v	11
hydrogen peroxide	0.15% v/v	70	0.078% v/v	25	0.0097% v/v	5	-----	

3.5. Broth microdilution assay of Clindamycin in combination with different antiseptics:

To assess the effect of Clindamycin/antiseptic combination on the MIC of Clindamycin against *P. acnes*, broth microdilution assay of Clindamycin alone and in combination with the best effective concentration of different antiseptics (as determined in 3.4) was carried out.

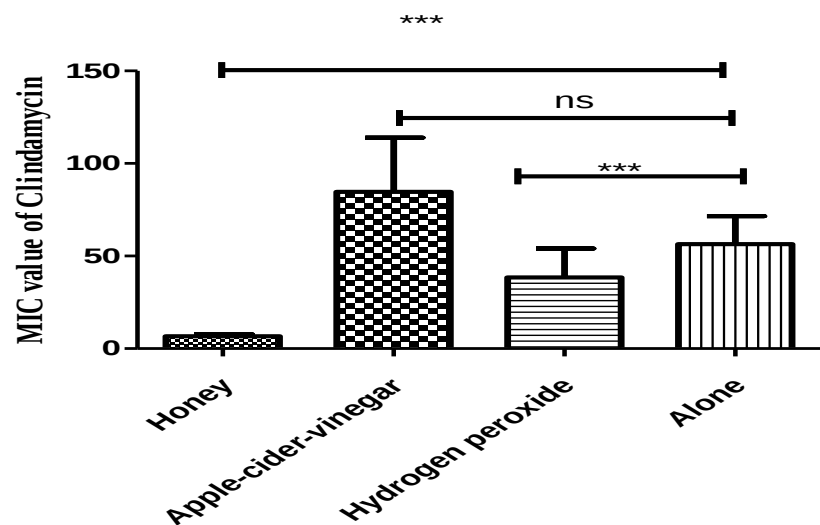


Figure3: MIC values of Clindamycin alone and after combination with 25% v/v honey, 1.25% v/v apple-cider-vinegar and 0.15% v/v hydrogen peroxide combination, against *P. acnes* isolates.

The results showed that MIC value of Clindamycin was significantly decreased after combination with 25% v/v honey and 0.15% v/v hydrogen peroxide, $P<0.05$, while no statistically significance difference between MIC value of Clindamycin alone and after combination with 1.25% v/v Apple cider vinegar against *P. acnes* isolates $P>0.05$.

Table 6: Synergistic and additive effect of Clindamycin plus honey, plus apple-cider-vinegar and (0.625%) hydrogen peroxide combination, and FIC index values.

	Synergism		Additive	
	%	FICI	%	FICI
Clindamycin plus honey	17/68 (25%)	Range (0.357-0.5) Mean 0.45	51/68 (75%)	Range (0.625-1.5) Mean 1.1
Clindamycin plus apple-cider-vinegar	2/68 (2.9%)	Range (0.357-0.5) Mean. 0.45	66/68 (97.1%)	Range (0.625-1.5) Mean 1.04
Clindamycin plus (0.15%) hydrogen peroxide	57/68 (83.82%)	Range (0.175-0.45) Mean 0.329	11/68 (16.17%)	Range (0.6-0.7) Mean 0.66

3.6 Checkerboard analysis

Checkerboard analysis was done to evaluate the effect of the candidate antiseptic combination with Clindamycin. Results illustrated combinations of Clindamycin plus (0.15%v/v hydrogen peroxide showed synergism in (57/68: 83.8%) of the *P.acnes* isolates. Clindamycin plus honey

resulted in synergistic effect in 25% of the *P. acnes* isolates. Additionally, Clindamycin plus honey showed (51/68:75%) additive effect, Clindamycin plus apple-cider-vinegar showed (66/68: 97.1%) additive effect. The mean of Fractional Inhibitory Concentration (FIC) index of each combination are presented in table 6.

Discussion:

The *P. acnes* has been implicated in the pathogenesis of acne vulgaris, through various mechanisms including bacterial colonization and causing inflammation (8). *P. acnes* is a fastidious anaerobic microorganism that requires special nutritional factors to grow (14). The present study examined 186 acne samples; 53 cases (28.5%) were found to be *P. acnes*-positive, using the culture methods and various specific biochemical tests including oxidase, indole, catalase, glucose and maltose fermentation and motility test (4,14). However, the identification of *P. acnes* using the culture technique is difficult and slow technique. Accordingly, molecular identification of *P. acnes* isolates, was carried out, to confirm the culture identification data. Molecular methods enable a greater diversity and higher percentage of the organisms to be identified. Polymerase chain reaction allows also identification of the unculturable (5,17), obligate anaerobic (5) and slow-growing organisms (17). Molecular identification of *P. acnes* isolates using 16S- rRNA gene revealed that 68 isolates (36.5%) were positive for amplification of 16S- rRNA gene of *P. acnes*. These results showing that PCR give higher level of identification, nevertheless, there was no statistically significant difference ($p > 0.01$) between the results obtained by the molecular technique (36.5%) and those obtained by the culture methods (28.5%). While Naghdi (2017) identified only 20% of *P. acnes* using the culture method, and 82.85% were identified using PCR, with $P < 0.001$ (18). These results might reflect our accuracy in handling the conventional biochemical tests.

Additionally, the ability of *P. acnes* to produce Hyaluronidases enzyme, which can degrade hyaluronic acid, represents one of its major virulence factors. The widespread presence of Hyaluronidases enzyme gene in *P. acnes* makes it a potential target for identification of *P. acnes* in various clinical specimens (7,19,20). Our results revealed that 57 out of 186 isolates were positive for amplification of hyaluronidase enzyme gene of *P. acnes*. These results may reflect the intensity of the disease, as these *P. acnes* were isolated from cases of acne vulgaris with moderate severity. Accordingly, future study which correlate the intensity of the disease and the level of expression of Hyaluronidases enzyme using real time PCR is recommended.

The use of antibiotics were the major approach in treatment of acne vulgaris, through inhibiting bacterial colonization, hence limiting inflammation (9). Misuse and overuse of systemic and topical antibiotics play a crucial rule in emerging of antibiotic resistance in *P. acnes* (8,21).

The present results showed that, *P. acnes* was highly sensitive to carbapenem antibiotic such as meropenem; penicillin derivative antibiotics including benzyl-penicillin and ampicillin and third-generation cephalosporin such as cefotaxime. Meanwhile, *P. acnes* showed higher resistance rates to certain macrolides antibiotics (such as Clindamycin, erythromycin) and specific cyclines antibiotics such as tetracycline. Normally, drug sensitivity is not requisitioned in the laboratories, due to the cost, the slow growth of *P. acnes* and complexity of the identification methods.

The present results are in consistence with other studies showing resistance to *P. acnes* against Clindamycin, erythromycin (8,9,21) and tetracycline (8). While Zhu, 2019 reported high sensitivity of *P. acnes* to cyclines including tetracycline, and Biswal (2016) reported sensitivity

of *P. acnes* to penicillin, these contradictory findings could be attributed to variations of geographical location, antibiotic usage and host factors. Also, the defined cyclines and macrolide antibiotics resistance may be explained by their widespread use. Clindamycin represents one of the most common drug prescribed in treatment of acne vulgaris, as it is used topically with no observable irritation in treatment of acne (9).

In order to overcome the emerging resistance of *P. acnes* against antibiotics, specifically, Clindamycin, we have tried to combine Clindamycin with other natural compounds of known antibiotic activity such as honey and apple cider vinegar; and chemical compounds such as hydrogen peroxide.

Using of hydrogen peroxide as an effective and safe alternative to Benzoyl peroxide is a novel approach in treatment of mild and moderate acne lesions (12). Both Benzoyl peroxide and hydrogen peroxide perform the bactericidal effect against *P. acnes* through production of oxygen free radicals and inhibition of bacterial colonization (22,23).

The combination of Clindamycin and Benzoyl peroxide showed clinical efficacy in the treatment of acne vulgaris, through its effect on *P. acnes*. The effect of such combination was significantly greater than the effect of each agent alone (24). Nevertheless, Benzoyl peroxide can cause skin dryness and irritation (12). Accordingly, in the present study we assessed the effect of Clindamycin and hydrogen peroxide combination against *P. acnes* using checkerboard analysis. The result illustrated that very low concentration 0.15% of hydrogen peroxide can show synergism in 83.82% of *P. acnes* isolates. Hydrogen peroxide has been reported to be safe and tolerable at concentration of 3% (25). Accordingly, hydrogen peroxide not expected to cause skin irritation at such very low concentration of 0.15%. However, the limitation of our study is that we did not perform chemical analysis of the combination of hydrogen peroxide and Clindamycin, to confirm its safety, so we recommend further chemical analysis of such combination in a future study.

In various studies, honey has been reported to have powerful antimicrobial properties, as it perform bactericidal and bacteriostatic action at higher and lower concentrations, respectively, with no disadvantage of developing antibiotic resistance (10,26,27). Noteworthy, a US patent for using honey specifically for acne treatment is exist (Mousa MA, 2001). Our results confirmed the effect of honey alone and in combination with Clindamycin, as it showed additive effect in 75% of *P. acnes* isolates.

Additionally, apple cider vinegar has a strong an anti-bacterial activity (29,30). It can inhibit *P. acnes* growth, and give different inhibition zones of inhibition of *P. acnes* at different apple cider vinegar concentrations (11). Our data showed inhibitory effect of apple cider vinegar alone on the growth of *P. acnes* isolates, while the data did not show any significance effect of apple cider vinegar on MIC value of Clindamycin against *P. acnes* isolates. Importantly, this combination showed additive effect in 97.2% of the isolates, with FICI 0.26.

Conclusion

To conclude, the present study suggests that the use of Clindamycin in combination with hydrogen peroxide may decrease the antibiotic resistance in *P. acnes*, lowering the MICs of Clindamycin. Therefore, alternative to the use of antibiotics alone, combination with hydrogen peroxide may not alter the antibiotic sensitivity of *P. acnes* in the treatment of acne vulgaris. To the best of our knowledge, this is the first study designed to overcome Clindamycin resistance of *P. acnes*, using combination of Clindamycin with low concentration of hydrogen peroxide.

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